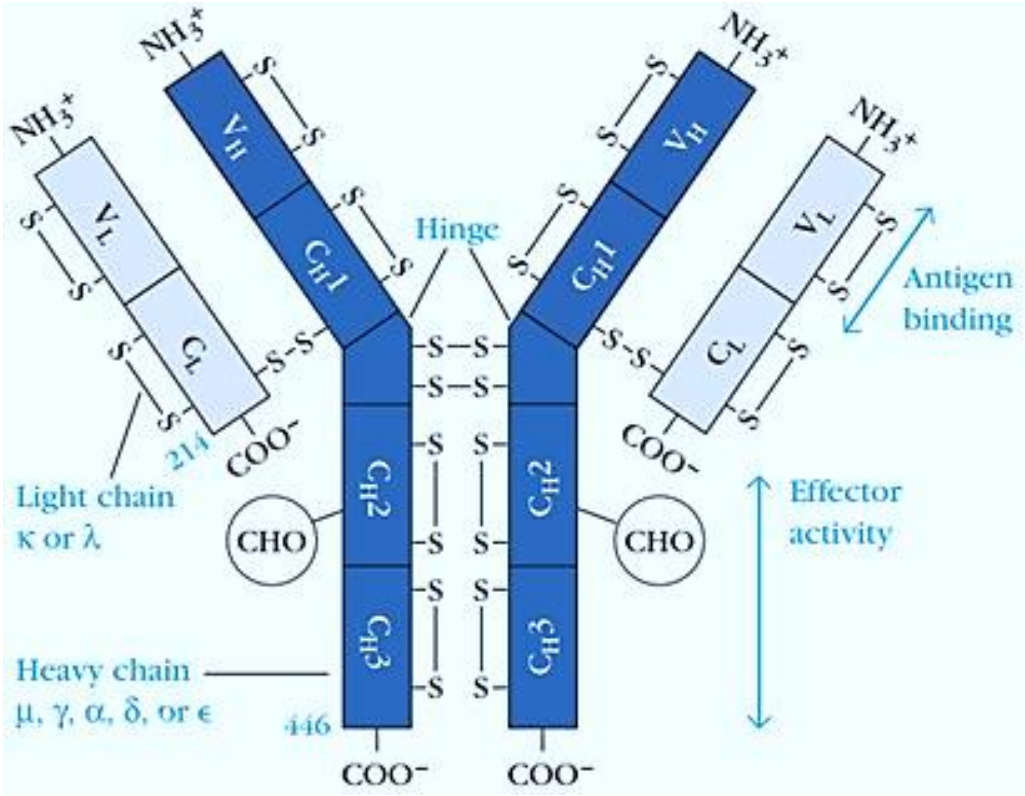
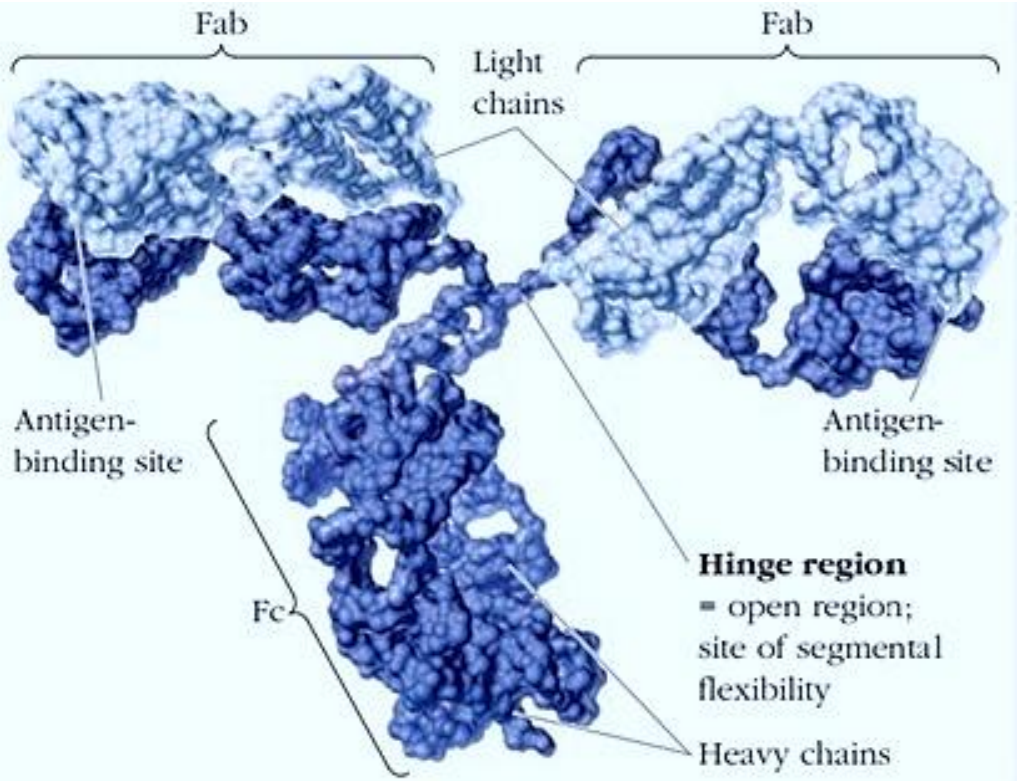




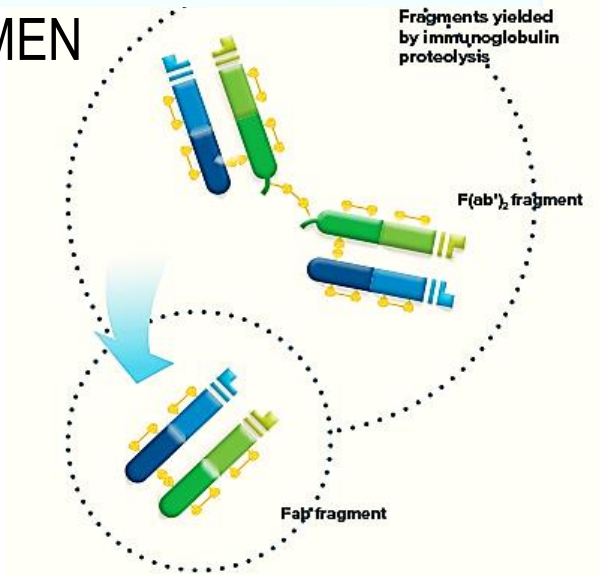
HIRALAL MAZUMDAR MEMORIAL COLLEGE FOR WOMEN

DAKSHINESWAR, KOLKATA-35

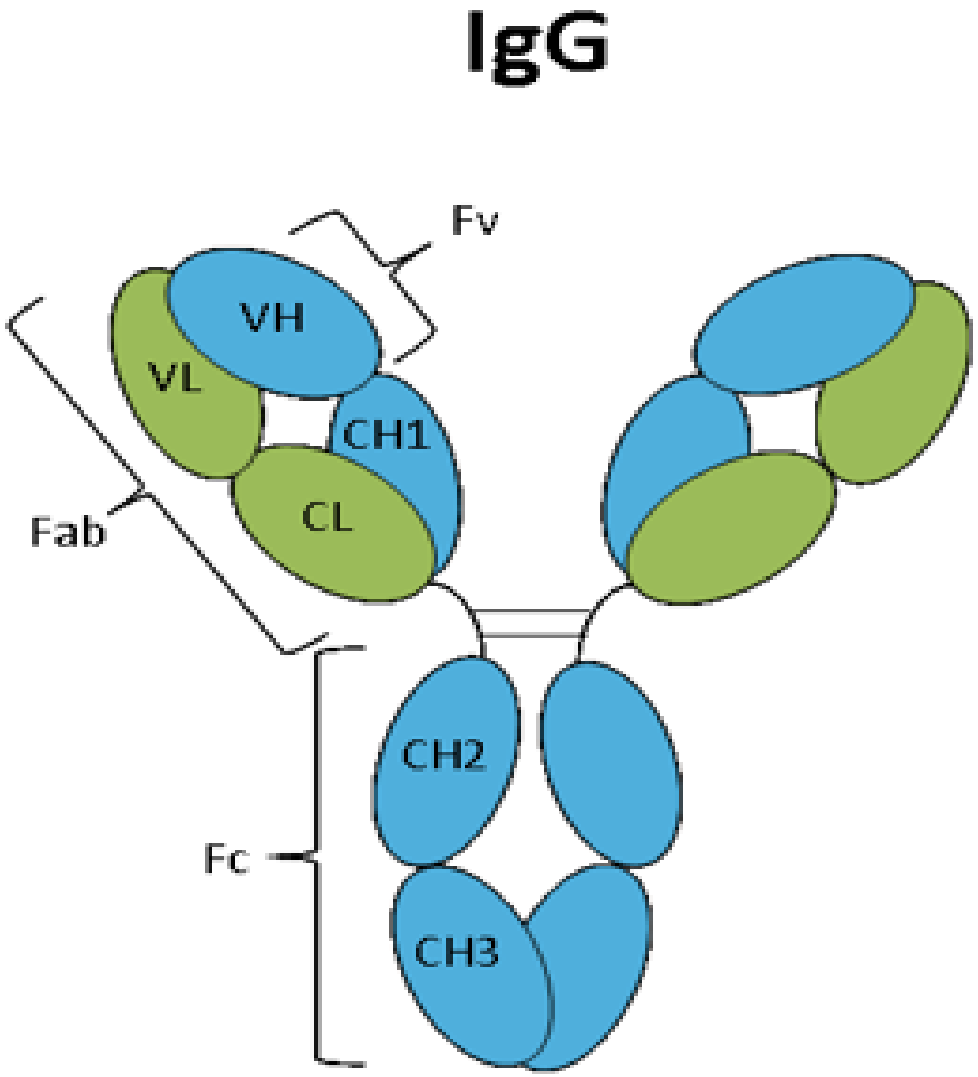
DEPARTMENT OF ZOOLOGY

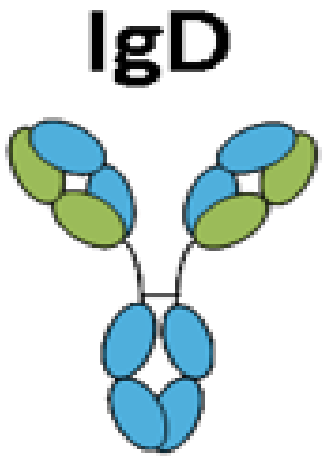
PRESENTATION BY *Rituparna Maity*

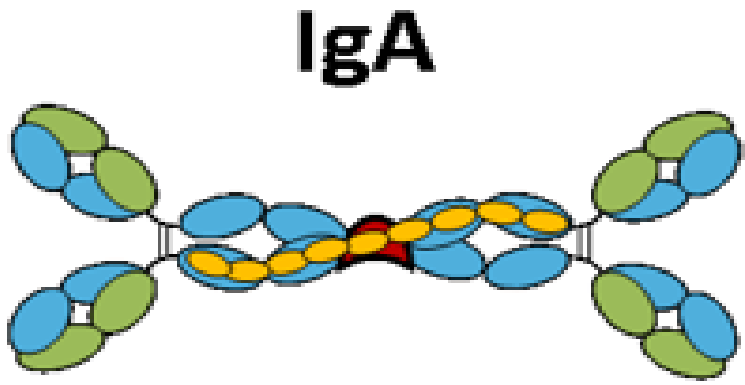
ZOOACOR10T: UNIT 05
IMMUNOGLOBULINS

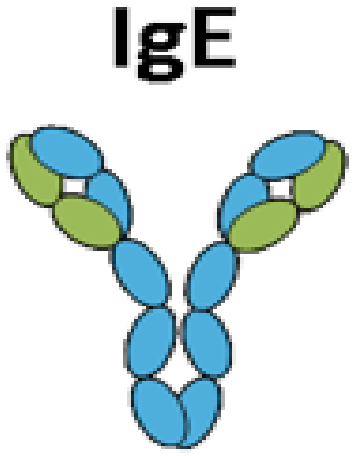


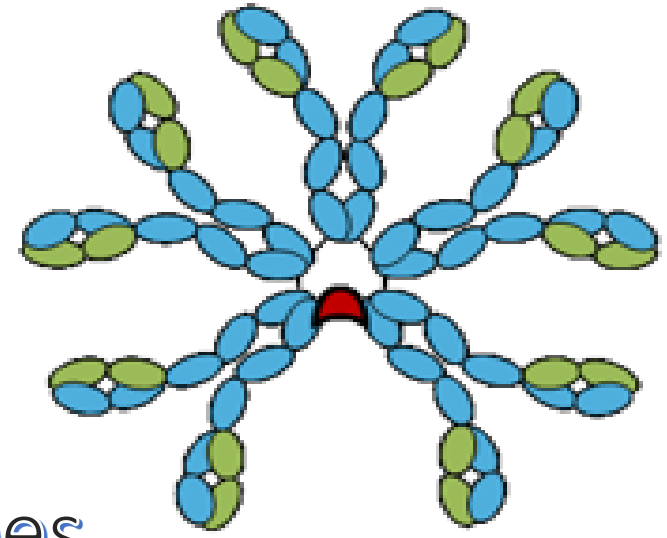
Immunoglobulin Classes/ Isotypes











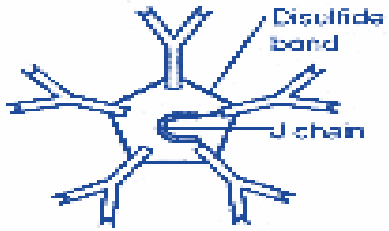
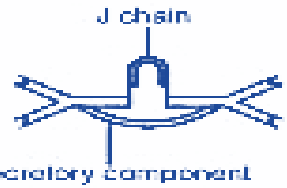
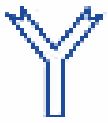
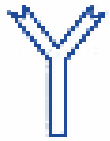
Heavy chain domain

Light chain domain

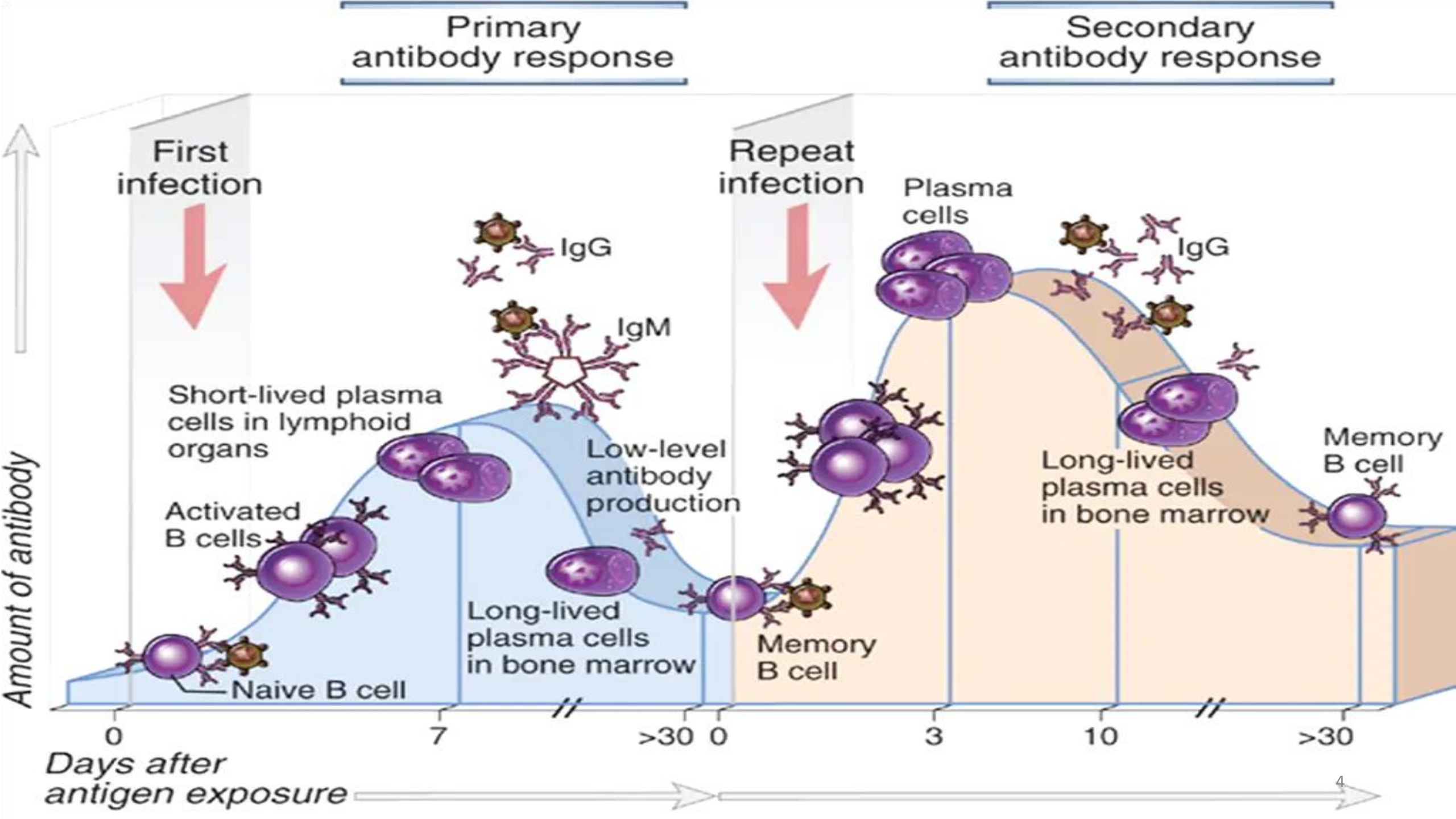
J chain

Secretory component

A Summary of Immunoglobulin Classes

Characteristics	IgG	IgM	IgA	IgD	IgE
					
Structure	Monomer	Pentamer	Dimer (with secretory component)	Monomer	Monomer
Percentage of total serum antibody	80%	5–10%	10–15%*	0.2%	0.002%
Location	Blood, lymph, intestine	Blood, lymph, B cell surface (as monomer)	Secretions (tears, saliva, mucus, intestine, milk), blood lymph	B cell surface, blood, lymph	Bound to mast and basophil cells throughout body, blood
Molecular weight	150,000	970,000	405,000	175,000	190,000
Half-life in serum	23 days	5 days	6 days	3 days	2 days
Complement fixation	Yes	Yes	No†	No	No
Placental transfer	Yes	No	No	No	No
Known functions	Enhances phagocytosis; neutralizes toxins and viruses; protects fetus and newborn	Especially effective against microorganisms and agglutinating antigens; first antibodies produced in response to initial infection	Localized protection on mucosal surfaces	Serum function not known; presence on B cells functions in initiation of immune response	Allergic reactions; possibly lysis of parasitic worms

*Percentage in serum only; if mucous membranes and body secretions are included, percentage is much higher.


Primary antibody response
Secondary antibody response
First infection
Repeat infection
IgG
IgM
Plasma cells
IgG
Short-lived plasma cells in lymphoid organs
Low-level antibody production
Activated B cells
Long-lived plasma cells in bone marrow
Memory B cell
Long-lived plasma cells in bone marrow
Memory B cell
Naive B cell
Amount of antibody
0
7
>30
0
3
10
>30
Days after antigen exposure

Schematically an Antigen-Antibody Reaction can be represented as:

Ag + Ab [Ag-Ab] → Aggregation → Precipitation/Agglutination/Neutralization

For diagnostic immunological tests, the serological tests must possess high specificity and sensitivity. Specificity is the ability of an antibody to recognize a single specific antigen. There is a high degree of specificity in antigen-antibody reactions.

Antibodies can distinguish differences in:

i. Primary structure of an antigen,

ii. Isomeric forms of an antigen, and

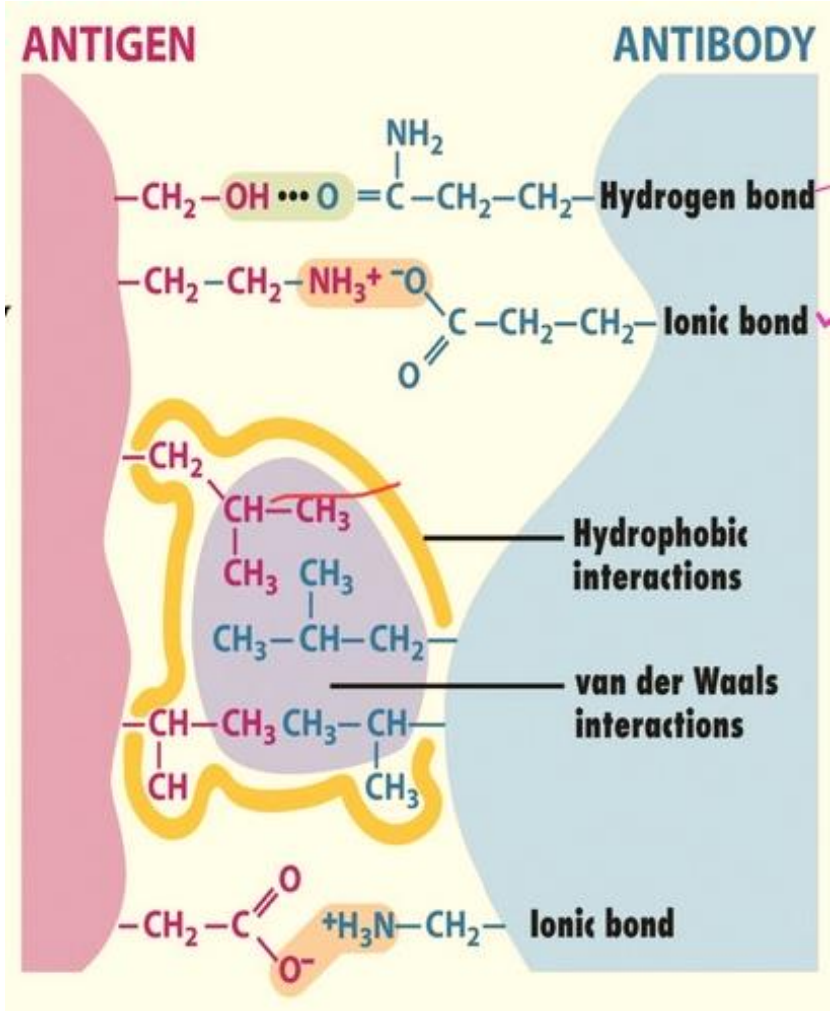
iii. Secondary and tertiary structure of an antigen.

Therefore specificity implies that:

a. Antibody is specific for a single and specific antigen.

b. Antibody wills not cross-react with other antigens.

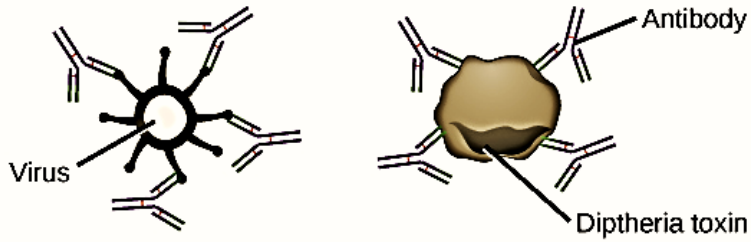
c. It will not give false positive results.



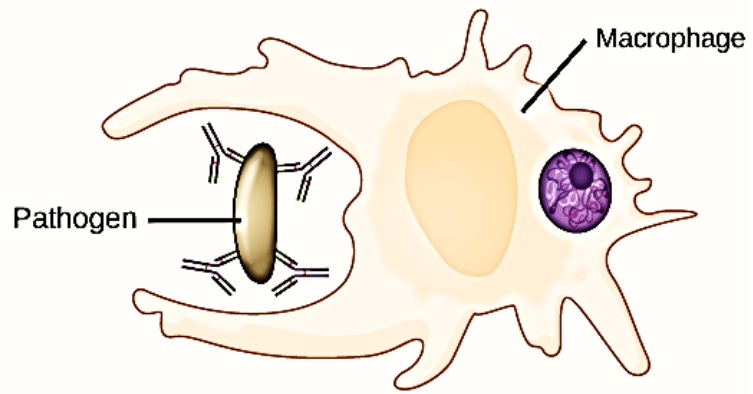
Antigen-Antibody Reactions-Biomolecular Reactions :

- Antigen (Ag) antibody (Ab) reactions occur when an antigen combines with a corresponding antibody to produce an immune complex. Therefore, an antigen-antibody reaction is thus a bimolecular association which is similar to an enzyme-substrate interaction but the only difference is that antigen-antibody reaction does not lead to an irreversible chemical interaction.
- The basis for antigen-antibody reactions are the non-covalent interactions like hydrogen bonds, ionic bonds, van der Waal interactions, hydrophobic interactions, etc. These interactions are individually weak, therefore, a large number of such interactions work together in an antigen-antibody reaction. The in vitro study of antigen antibody reactions is known as **serology**.
- The main use of antigen-antibody reactions is in the determination of blood groups for transfusion, serological ascertainment of exposure to infectious agents, and development of immunoassays for the quantification of various substances.
- The principle for all diagnostic immunological tests is serological reactions. The binding of an antibody with an antigen of the type that stimulated the formation of the antibody, results in agglutination, precipitation, complement fixation, greater susceptibility to ingestion and destruction by phagocytes, or neutralization of an exotoxin.
- Sensitivity means the lowest amount of antigen that can be detected. If in a diagnostic test an antibody is capable of detecting a single antigen molecule, then such a test possesses highest sensitivity. The amount of antigen detected in a test is directly proportional to the amount of antibody used. **Enzyme Linked Immuno Sorbent Assays (ELISA) is the most sensitive serological tests.**

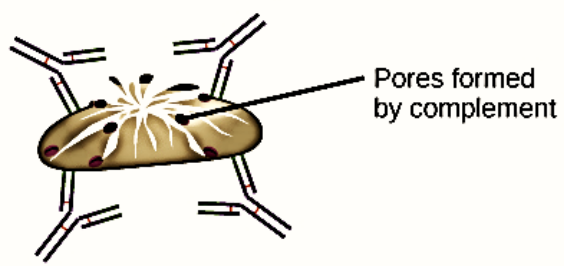
(a) Neutralization Antibodies prevent a virus or toxic protein from binding their target.



(b) Opsonization A pathogen tagged by antibodies is consumed by a macrophage or neutrophil.



(c) Complement activation Antibodies attached to the surface of a pathogen cell activate the complement system.



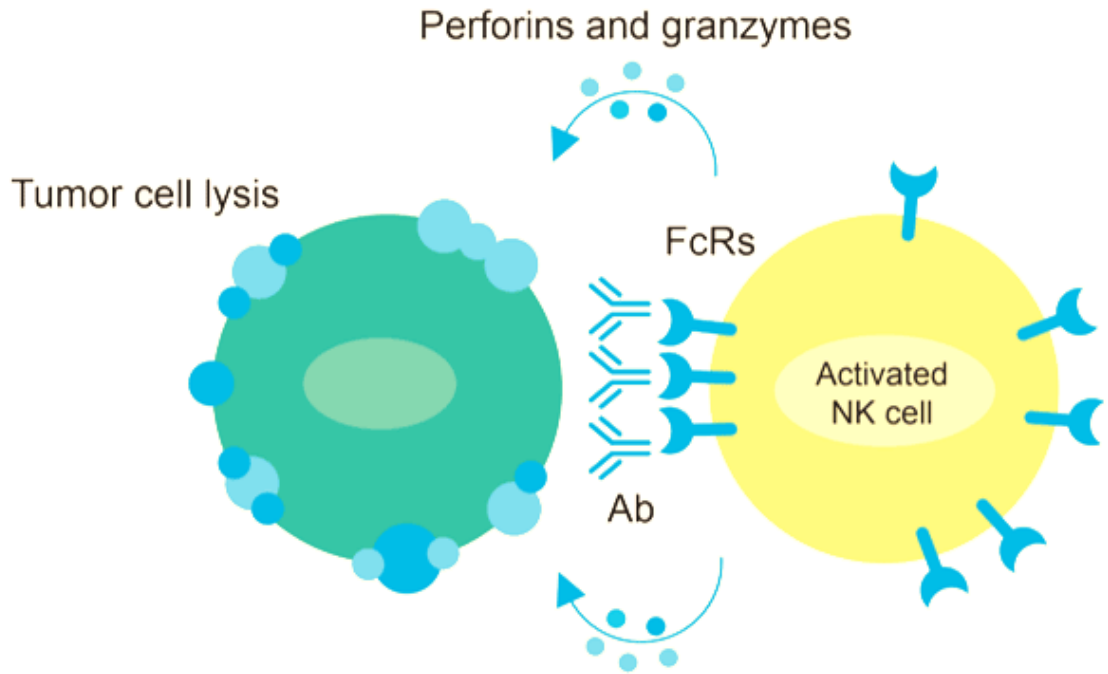


Figure 1: Antibody-Dependent Cellular Cytotoxicity (ADCC)

Antigen- Antibody Interactions- Types:

1. Neutralization: Certain antibodies called neutralizing antibodies react with antigen and neutralize them so that they fail to attach on host cell surface.

2. Immune Complex Formation /Agglutination & Precipitation: Antibodies possess at least two antigen-binding sites and most antigens have at least two epitopes (antigenic determinants). The antibodies cross-link antigens forming large aggregates of antibody and antigen referred to as immune complexes (Fig. 41.17), which are more readily phagocytized than are free antigens.Depending upon their physical properties, immune complex forming antibodies are of two types: precipitins and agglutins. The precipitins react with antigens that are soluble molecules and form immune complex large enough to precipitate: this process is called precipitation (L. praecipitare = to cast down) or precipitin reaction.The agglutins, however, react with surface-bound antigens of bacterial or other cells and form immune complex. This process is called agglutination or agglutin reaction. Agglutination specifically involving red blood cells is called hem-agglutination and is caused by antibodies called hemagglutin.

3. Opsonization: Opsonization (G. opson = to prepare victim for) or opsonin-dependent recognition is one of the two fundamental molecular mechanisms used by phagocytic cells for the recognition of microbial pathogens and their adherence on phagocyte's plasma membrane so that the phagocytes can ingest them easily.

4. Complement System: Complement fixation refers to the ability of antigen-antibody-complex to bind complement so that the latter becomes "fixed" and "used up". It is operated by a system called the complement system which consists of over 30 soluble and cell-bound proteins and glycoproteins that interact in a highly regulated cascade. This system involves in antigen-antibody interaction in host to play its role in immune response.

5. Antibody-Dependent Cell-Medicated Cytotoxicity (ADCC): Many cells that have cytotoxic potential express membrane receptors for the Fc region of the antibody molecule. When antibody is specifically bound to a target cell, the receptors of these cells can bind to the antibody Fc region. Thus the cytotoxic cells bind to the target cells, and subsequently cause lysis of the target cells. Although these cytotoxic cells are nonspecific for the antigen, the specificity of the antibody bound to the target cells directs the cytotoxic cells to specific target cells. This type of cytotoxicity is referred to as antibody- dependent cell-mediated cytotoxicity (ADCC). The cells that can mediate ADCC are NK cells, macrophages, monocytes, neutrophils, and eosinophils. ADCC appears to involve a number of different cytotoxic mechanisms, but it does not involve complement-mediated lysis

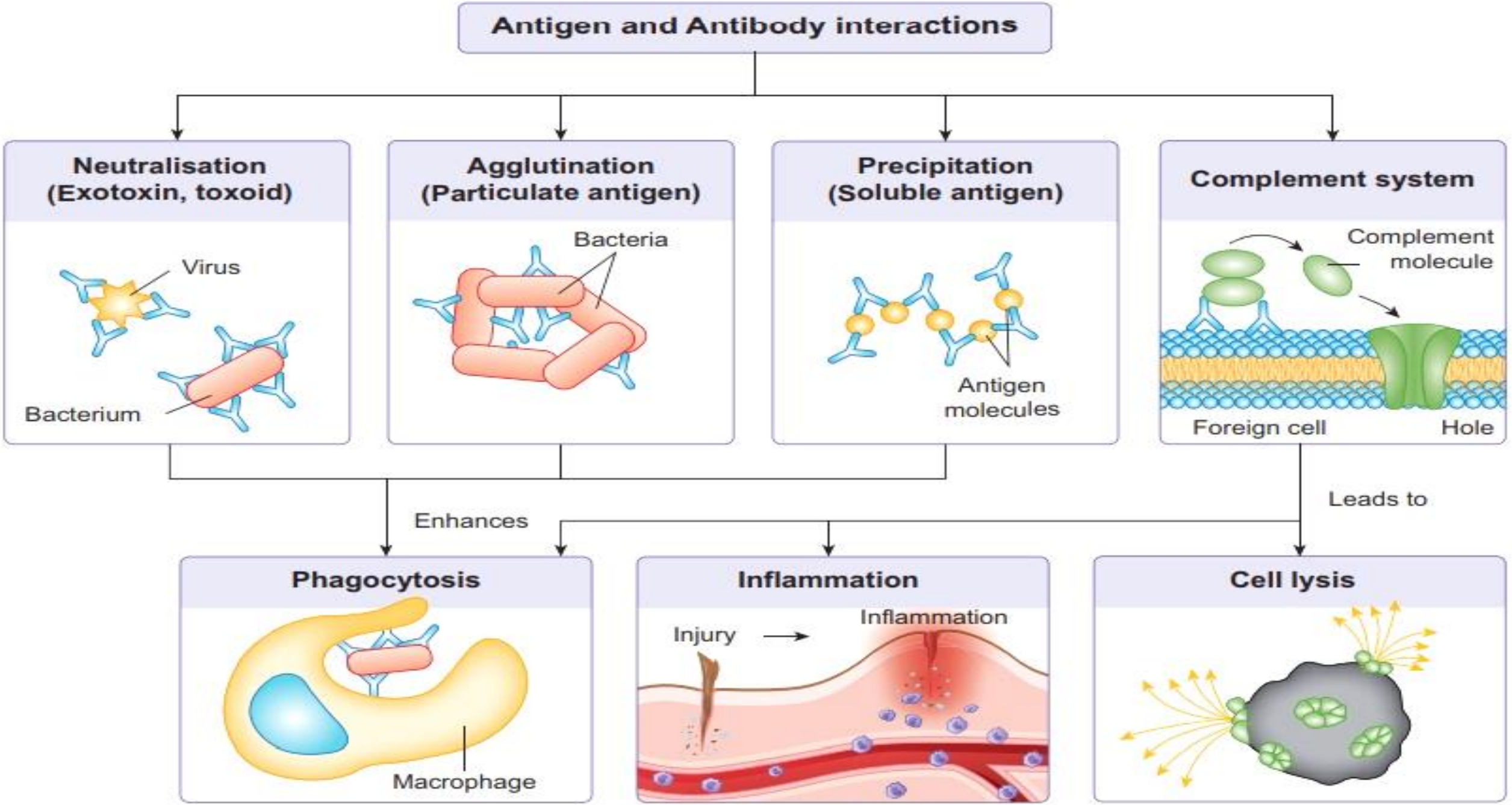
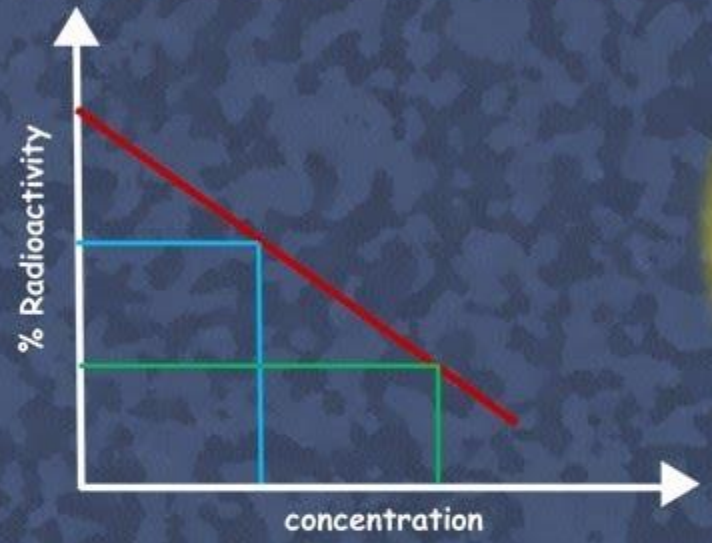
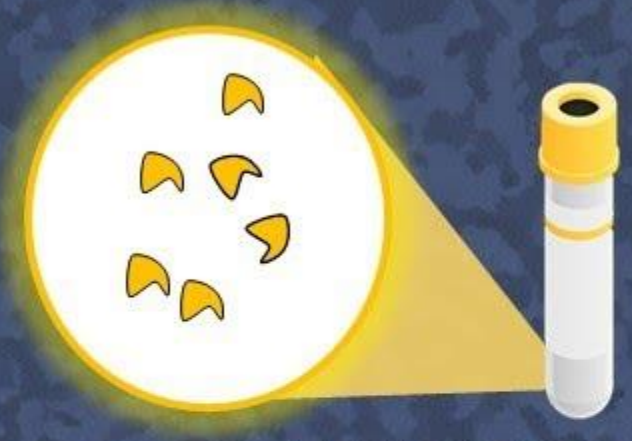


Fig. 8.8 Antigen and antibody reaction

Radioimmunoassay (RIA)





Unlabelled antigen
(from patient serum)

A antigen

Anti A antibody

Radioimmunoassay (RIA) uses antibodies to detect and quantitate the amount of antigen (analyte) in a sample.

RADIOIMMUNOASSAY/ RIA : One of the most sensitive techniques for detecting antigen or antibody is Radio-Immuno Assay (RIA)

- **History:**The technique was first introduced by two endocrinologists, S. A. Berson and Rosalyn Yalow, in 1960. It was first used to determine the levels of insulin-anti-insulin complexes in diabetics. After a lot of controversy, the technique was proved as an effective one for quantification of hormones, serum proteins, drugs and vitamins even at concentrations of 0.001 µg or less. In 1977, the significance of the technique of RIA was acknowledged by the award of a Novel Prize to Yalow, which was after the death of Berson.
- **Principle:**RIA is a highly sensitive method of estimation of antigens or haptens. The principle of RIA is a competition between a radioactive antigen or a hapten (a ligand) and a non-radioactive counterpart of the same antigen or hapten for binding to the sites of the cognate antibody molecules. The antigen is generally labelled with a gamma- emitting isotope as for e.g.125I.
- Technique of RIA is of two types: (i) Direct RIA and (ii) Indirect RIA.
- **Direct RIA:** When antigens or primary antibodies are directly labelled with a radionuclide, it forms the basis of Direct RIA. This technique utilizes radiolabelled antibody or its ligand (antigen). Antibody is incubated with ligand and unbound reactants are removed from the system (phase separation).It may utilize precipitation of bound reactants (quantitative precipitin reaction), particulate antigens (such as bacteria), the immobilization of the nonradioactive reactant onto a solid matrix (such as plastic), and so on.
- **Indirect RIA:** Anti-immunoglobulin antibody (secondary antibody) is radio-labelled and used in the indirect RIA. This technique uses radiolabeled secondary antibody (anti-immunoglobulin) to detect the binding of a primary antibody.
- **Methods of quantification by RIA**: (i) The labelled antigen is mixed with antibody at a concentration that just saturates the antigen-binding sites of the antibody molecules and then increasing amounts of un-labelled antigen of unknown concentration are added. (ii) The antibody does not distinguish labelled from un-labelled antigen for which two kinds of antigen compete for available binding sites on the antibody. (iii) Gradually, the concentrations of un-labelled antigen is increased and more labelled antigen will be displaced from the binding sites. (iv) Now, the decrease in the amount of radio-labelled antigen bound to specific antibody in the presence of test sample is measured in order to determine the amount of antigen present in the test sample.

Reaction of RIA:

In RIA, the essential components of the analytical system are the antigen (Ag) to be determined, a fixed amount of labelled antigen (Ag*) and a fixed limited amount of antibody (Ab).

The reaction is: $Ag + Ag^* + Ab \rightleftharpoons AgAb + Ag^*Ab$

The most important of the immuno-enzyme assays are the Enzyme-linked immunosorbent assays, commonly called ELISA. It is a type of solid-phase enzyme immunobinding assay. In ELISA antigen is linked to a solid phase anchored antibody in such a way that retains both immunological and enzymatic activity. The solid phase may be of polystyrene/polyvinylchloride.
So ELISA can be called as a qualitative or quantitative assay for antibodies, an assay for quantitating either antibody or antigen by use of an enzyme-linked antibody and a substrate that forms a coloured reaction product.

- **Principle:**The basic principle of ELISA depends on the presence of enzyme. An enzyme conjugated to an antibody reacts with a colourless substrate to generate a coloured reaction product. ELISA is similar to RIA in principle, depends on an enzyme but avoiding the involvement of radioactive label.
- **Requirements:** (i) Antigen coated microtiter well (ii) Primary antibody (Ab1) (iii) Secondary antibody (Ab2) remain conjugated with enzyme (iv) Specific enzyme : alkaline phosphatase, horseradish peroxidase and β-galactosidase (v) Specific substrate(vi) Suspected blood sample (serum).
- **Types:** A number of variations of ELISA have been developed. Each type of ELISA can be used qualitatively to detect the presence of antigen/antibody. **I. Indirect ELISA II. Sandwich ELISA III. Competitive ELISA**
- **Indirect ELISA:** Antibody can be detected or quantitated with an indirect ELISA.
- **Steps involved:** 1. Serum or some other sample contains primary antibody (Ab1) is added to an antigen- coated microtiter well and allowed to react with the bound antigen. 2. After reactions, free Ab1 is washed away and antigen bound antibodies are present within the microtiter well. 3. Addition of enzyme conjugated secondary antibody (Ab2) to the microtiter well which will bind with the primary antibody. 4. After sometime, a wash is done to remove excess Ab2 from the set up. 5. Now, specific substrate for specific antibody is added and as a result, a coloured reaction product is formed. 6. The coloured reaction product is measured by specialized spectro-photometric plate reader.
- **Sandwich ELISA:** Antibody can be detected or quantitated by a sandwich ELISA.
- **Steps involved:**1. The antibody (Ab1) is placed (immobilized) on a microtiter well. 2. A sample contains antigen is added and allowed to react with the bound antibody (Ab1). 3. A wash is taken to remove excess free antibody from the well. 4. After that, a second antibody (Ab2) specifically bound with enzyme is added which binds with different epitope present on the bound antigen. 5. A second wash is needed to remove free second antibody. 6. Again a specific substrate is added and the coloured reaction product is measured.

- **Competitive ELISA**: Another variation for measuring amounts of antigen is Competitive ELISA.

  Steps involved: 1. In this technique antibody is first incubated in solution with a sample containing antigen. 2. The antigen-antibody mixture which is thus formed, is then added to an antigen-coated microtiter well. 3. The more antigen present in the sample, the less free antibody will be available to bind to the antigen-coated well. 4. An enzyme conjugated secondary antibody (Ab2) specific for the iso-type of the primary antibody is added. 5. Now, primary antibody is used to quantitate the amount of primary antibody bound to the well. 6. Colour changes are being monitored. More the colour is observed less will be the analyte in the test specimen, i.e., in the competitive assay, the higher will be the concentration of antigen in the original sample, indicates the lower absorbance .

What is a "Sandwich" assay?

It is a popular modification of a quantitative solid-phase assay where two antibodies; specific to two different epitopes of the same antigen, are allowed to react with the antigen. Here, antigen is present within two layers of antibody like butter between two pieces of bread in a sandwich.

Ab_1—Antigen—Ab_2—Enzyme-linked

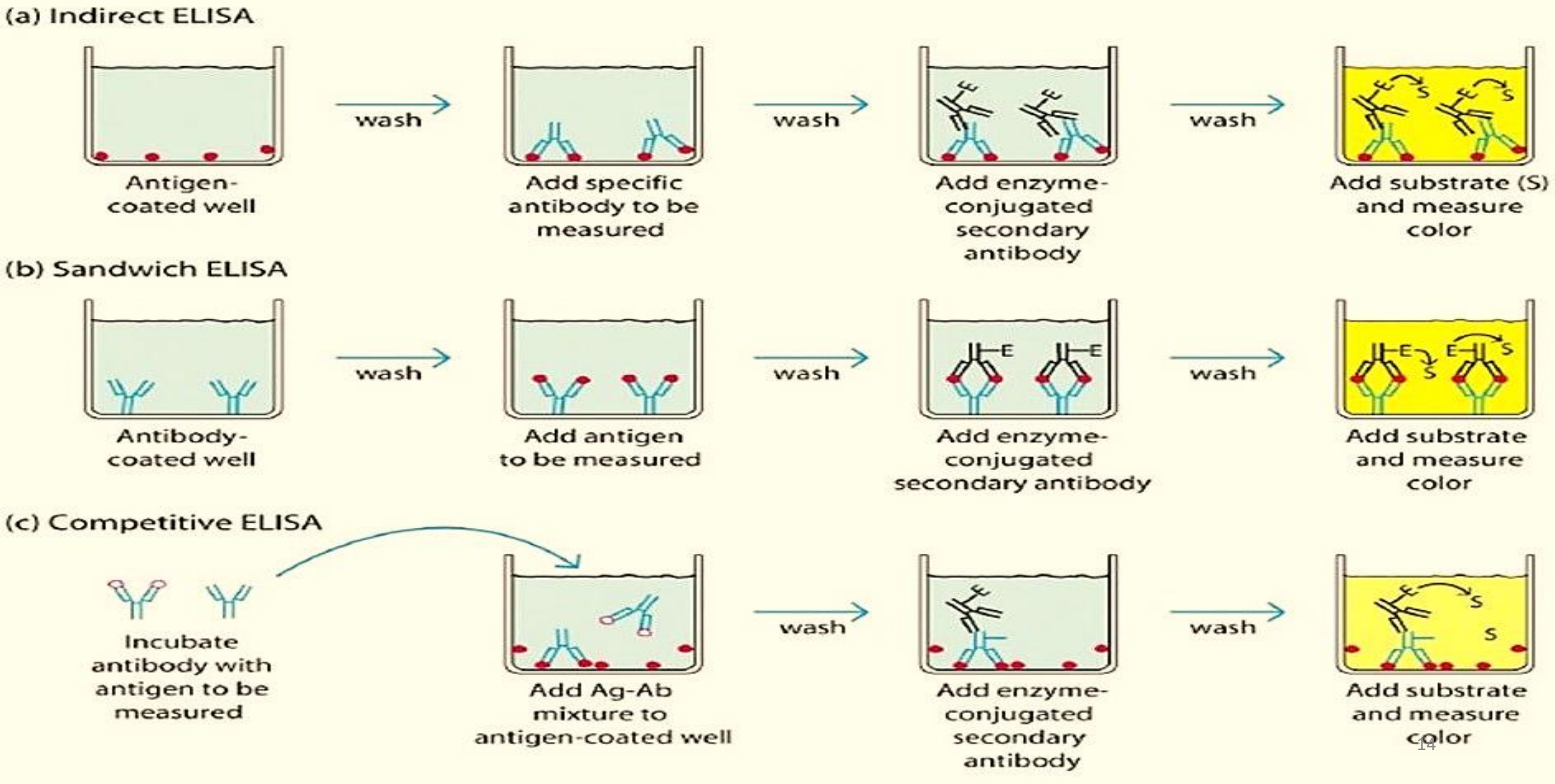
↓ ↑ Addition of substrate

Coloured product

What are the advantages of indirect and sandwich assays?

Indirect solid-phase immunoassays employ secondary antibodies that are versatile and able to detect species specific-antibodies of all specificities. In both the cases, they have a much higher sensitivity. The lower detection limit of ELISA is between 10-100 picograms.

Types of ELISA



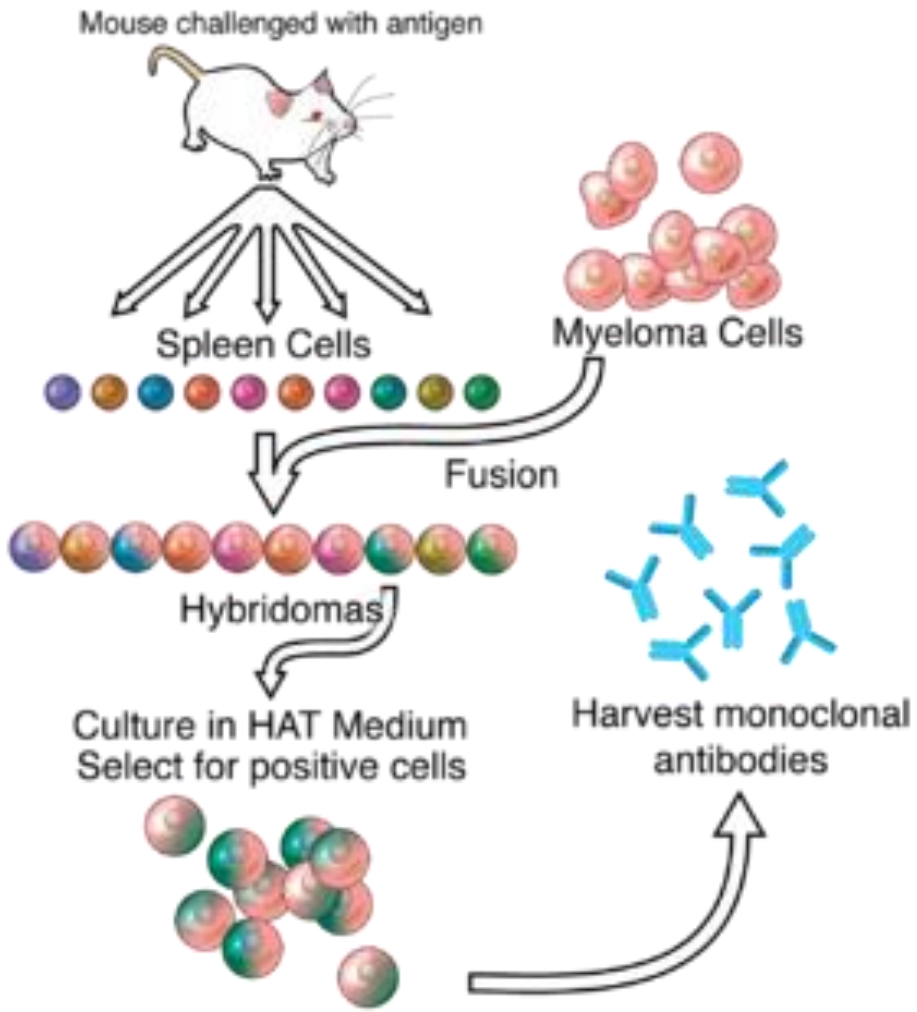
Monoclonal antibody (MAb) is a single type of antibody that is directed against a specific antigenic determinant (epitope). It was a dream of scientists to produce MAbs for different antigens. In the early years, animals were immunized against a specific antigen, B-lymphocytes were isolated and cultured in vitro for producing MAbs. This approach was not successful since culturing normal B-lymphocytes is difficult, and the synthesis of MAb was short-lived and very limited.

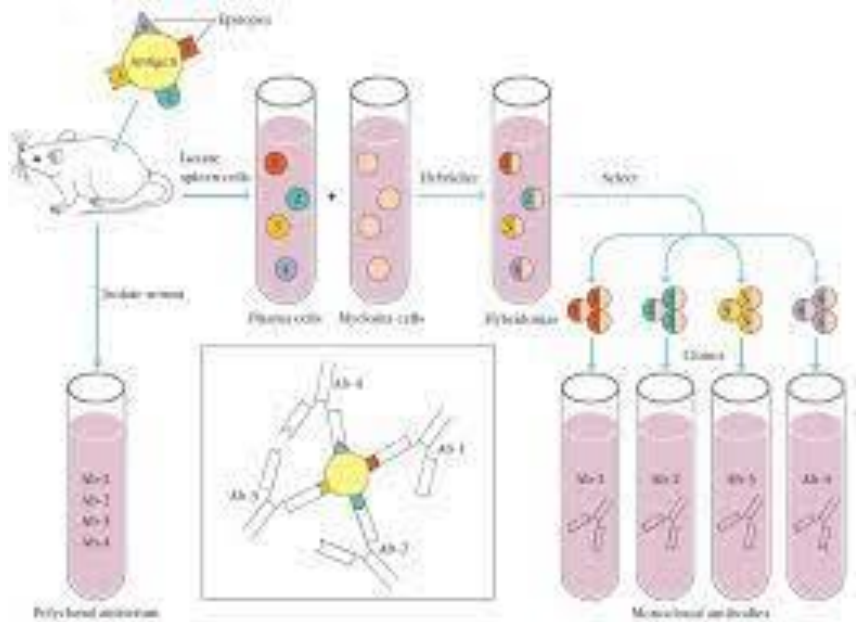
George Kohler and Cesar Milstein (Nobel Prize, 1984) achieved large scale production of MAbs. They could successfully hybridize antibody—producing B-lymphocytes with myeloma cells in vitro and create a **hybridoma.** The result is that the artificially immortalized B-lymphocytes can multiply indefinitely in vitro and produce MAbs. The hybridoma cells possess the growth and multiplying properties of myeloma cells but secrete antibody of B-lymphocytes. The production of monoclonal antibodies by the hybrid cells is referred to as **hybridoma technology.**

- **Production:**

1. A mouse is injected with a specific antigen that will induce antibodies against that antigen.
2. The spleen of the mouse is removed and a suspension is made. The suspension includes B cells that produce antibodies against the injected antigen.
3. The spleen cells are then mixed with myeloma cells (cancer cells) that are capable of continuous growth in culture but have lost the ability to produce antibodies. Some of the antibody-producing spleen cells and myeloma cells fuse to form hybrid cells. These hybrid cells are now capable of growing continuously in culture while producing antibodies.
4. The mixture of cells is placed in a selective medium that allows only hybrid cells to grow.
5. Hybrid cells proliferate into clones called hybridomas. The hybridomas are screened for production of the desired antibody.
6. The selected hybridomas are then cultured to produce large quantities of monoclonal antibodies (MAbs) because they come from a single clone of identical cells.

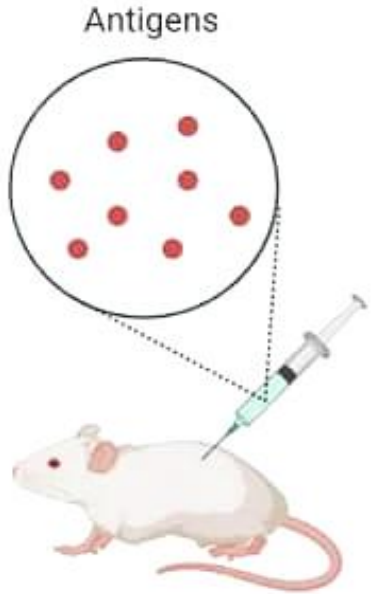
- **Uses:** Uses of monoclonal antibodies include the following. They recognize several bacterial pathogens, diagnosis of pregnancy, allergies and diseases such as hepatitis, rabies and some sexually transmitted diseases. MAbs have also been used to detect cancer at an early stage and to know the extent of metastasis.
- MAbs are also being used since 1986 to minimize rejection of kidney transplants. For these purposes MAbs are prepared that react with the T cells that are responsible for rejection of the transplanted tissue. The MAbs suppress the T cell activity. They may also be used to treat autoimmune diseases.


Mouse challenged with antigen
Spleen Cells
Myeloma Cells
Fusion
Hybridomas
Culture in HAT Medium
Select for positive cells
Harvest monoclonal
antibodies

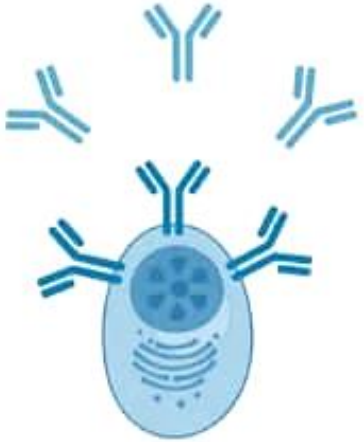


Hybridoma: Because of the highly specific nature of the antibodies produced by differentiated B lymphocytes, cultures of the many lines of these cells have long been contemplated as a source of antibodies for clinical application.
Whereas the highly differentiated B lymphocytes cannot be cultured, hybrid cells formed by fusing malignant myeloma cells with B lymphocytes do proliferate in culture and synthesize their respective antibodies. These hybridomas can be separately cloned to yield large cultures of cells producing specific antibodies. Antibodies produced by this process are called monoclonal antibodies.

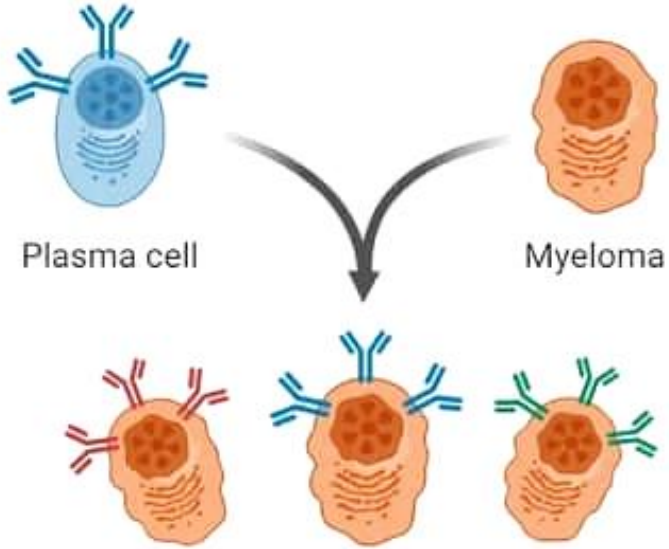
- In 1975, C. Milstein and G. Koehler were able to fuse myeloma cells with normal B lymphocytes, thereby producing a hybrid cell that could be grown in culture. Moreover, the hybrid cell, called a **hybridoma,** produces and secretes antibodies characteristic of the clone from which the normal lymphocyte is taken. First, an immune response is induced in a normal animal by exposure to a specific antigen (either by injecting the purified antigen or by injecting bacteria or viruses).
- Lymphocytes are then obtained from the animal's spleen or other lymphoid tissue. Among the cells that are removed will be some from clones that were activated by exposure to the antigen (many, perhaps most, of the cells will be from clones not selected by the antigen).
- The normal lymphocytes are then mixed with myeloma cells in a solution of polyethylene glycol. The myeloma cells to be used lack the enzyme hypoxanthine-quanine phosphoribosyl transferase (HGPRT), which catalyzes the synthesis of inosine monophosphate (IMP) and guano- sine monophosphate (GMP). Polyethylene glycol induces fusion of the two families of cells, thereby forming hybridomas.
- Some of the hybridomas that are formed will be hybrids of myeloma cells and the antigen-activated normal lymphocytes. Of course, the polyethylene glycol solution will also contain a number of unfused myeloma cells and normal lymphocytes.
- The un-fused lymphocytes will fail to grow (or grow so slowly that they produce insignificant numbers of progeny) when the cells are subcultured. If subculturing is carried out in a medium containing hypoxanthine, aminopterin, and thymidine (i.e., HAT medium), un-fused myeloma cells will also die out because they cannot produce HGPRT.
- The hybridomas will proliferate because the lymphocyte nuclei contribute functional HGPRT genes to the heterokaryon. The particular hybridomas producing the desired antibody can be identified using a suitable assay procedure, and these can be selected and separately cloned. Thus, the end result is a culture of cells producing a single type of antibody—a monoclonal antibody. Such cultures can be maintained indefinitely and be used as a continuous source of antibody.



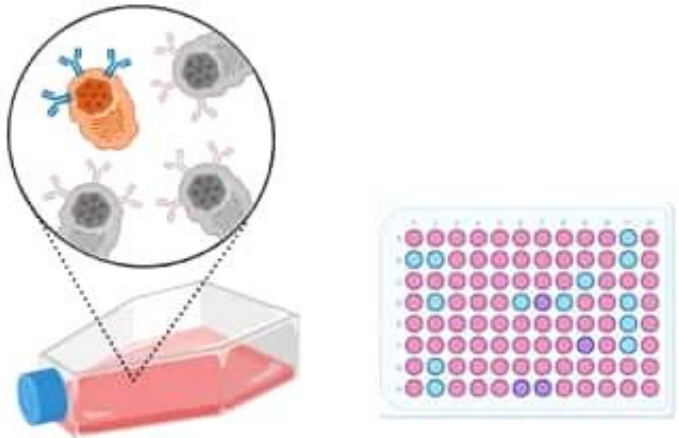
1 Immunization of mice with cancer specific antigens to stimulate antibody production



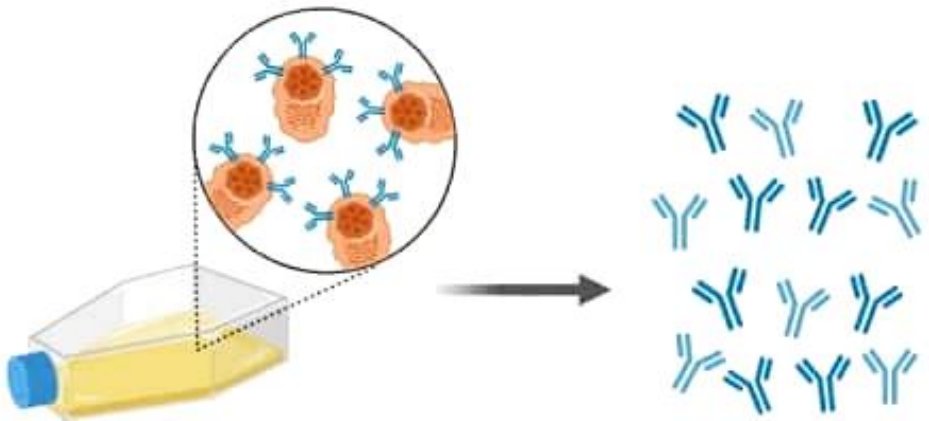
2 Isolation of antibody secreting plasma cells



3 Fusion and generation of hybridomas



4 Selection in HAT medium and ELISA screening



5 Expansion of selected hybridoma to produce monoclonal antibodies